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# Method for the construction of randomized gene sequence libraries in cells

## Technical Field

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The present invention provides an *in vivo* method for the generation of randomized gene libraries by means of homologous recombination wherein the *Kluyveromyces lactis* killer toxin is used as negative selectable 10 marker.

## Background Art

For functional studies, it is crucial to ana- 15 lyze individual regions of a protein systematically. This is usually performed by randomizing all amino acids of the region or by varying few conserved positions depending on the size of the region of interest. On one hand, such data results in the identification of key amino ac- 20 ids determining the interaction of a protein region for example with another protein region, with DNA, or with lipid structures. On the other hand, expression in a cellular environment of either a randomized protein region alone or of a randomized region in the context of a full 25 protein directly contributes to the understanding of the protein's function. Another application of region randomization is changing the binding properties of a single chain antibody (scFv). A scFv consists of variable regions of both the light and heavy chain. Each chain fea- 30 tures three variable regions, called CDR1, CDR2, and CDR3. Of the six CDRs, the CDR3 of the heavy chain is the major determinant of the scFv's affinity for its antigen. For changing the binding properties of a scFv, the heavy chain CDR3 region of a scFv with a given interaction pro- 35 file is randomized and screened against the epitope of interest. This usually leads to the identification of scFvs with a low affinity. For improving the affinity,

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the light chain CDR3 of the novel scFv is then random-
ized. Therefore, stepwise randomization of two scFv vari-
able regions can be used to isolate new or better binders
against a given epitope.

5          The classical protocol for region replace-
ment/randomization in the context of library production
is performed usually in bacteria such as *E. coli*. It in-
volves production of PCR fragments representing the li-
brary of regions, digestion with restriction enzymes cut-
10  ting at or close to the end of the fragments of interest,
and ligation into a vector linearized with matching re-
striction enzymes.

          For use in yeast, libraries have been pro-
duced directly in yeast with homologous recombination
15  (Hua, Luo et al. 1998). The process requires production
of a donor DNA fragment bearing on each end homologies to
the linearized target vector. Fragments for use in ho-
mologous recombination can therefore be produced by PCR
with primers bearing the homologous sequences at their 5'
20  ends.

          US Patent application published under No.
US2002/012734 describes a method for directed evolution,
gene reassembly and directed mutagenesis using homologous
recombination and selectable markers for counterselection
25  in bacteria.

          Although there exist already methods for the
construction of randomized gene sequence libraries by
means of homologous recombination in cells, there is
still a need for methods which allow an efficient con-
30  struction and selection of such randomized libraries.


          Disclosure of the Invention


35          Hence it is a general object of the present
invention to provide a method for the construction of

randomized gene libraries in cells. Said method comprises the following steps:

    introducing into suitable host cells capable of homologous recombination

5        a) a target vector comprising at least a first DNA sequence coding for at least a $\gamma$-subunit of a *Kluyveromyces lactis* killer toxin as negative selection marker, said DNA sequence being flanked at its 5' end by

10        a first target sequence and at its 3' end by a second target sequence and

        b) a donor DNA sequence which is flanked at its 5' end by a DNA sequence which is homologous to said first target sequence and

15        flanked at its 3' end by a DNA sequence which is homologous to said second target sequence and

cultivation of said host cells under suitable conditions allowing the selection of host cells in which said DNA

20 sequence in the target vector encoding at least a $\gamma$ - subunit of a *K. lactis* killer toxin has been replaced by said donor sequence by means of homologous recombination thereby abolishing expression of said $\gamma$ -subunit of a *K. lactis* killer toxin.

25    In a preferred embodiment said target vector further comprises a second DNA sequence which encodes at least one protein region, preferably more than two protein regions of a protein of interest, more preferably a full length protein.

30    In a further preferred embodiment said first DNA sequence of the target vector which comprises at least the coding region of the $\gamma$-subunit of the *K. latics* killer toxin and said two flanking regions, replaces a DNA sequence of said second DNA sequence which encodes a

35 protein region.

    In another preferred embodiment of the present invention, said DNA sequence encoding at least the $\gamma$

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subunit of the *K. lactis* killer toxin is under transcriptional control of a heterologous promoter, preferably a constitutive promoter, more preferably a *TEF* promoter from Ashbya gossypii.

In a further preferred embodiment said promoter is located between the DNA sequence encoding at least the γ-toxin subunit of *K. lactis* killer toxin and one of the two target sequences for homologous recombination.

In a further preferred embodiment said first DNA sequence of said target vector comprises a unique recognition site for a restriction enzyme. Said unique recognition site is preferably located in the coding region of the γ-toxin DNA sequence or more preferably between the coding region of the γ-toxin DNA sequence and the promoter driving transcription of the γ-toxin subunit.

In still another preferred embodiment said second DNA sequence of said target vector encodes an antibody or a single chain antibody (scFv).

In a much preferred embodiment of the present invention said first DNA sequence of said target vector replaces a DNA sequence in said second DNA sequence of said target vector which comprises at least the coding region of a CDR region of an antibody or a single chain antibody, preferably a CDR3 region, more preferably a CDR3V$_L$ region, even more preferably a CDR2 and a CDR3 region.

A further preferred embodiment relates to a method in which said first DNA sequence of said target vector comprising at least the γ-subunit of a *K. lactis* killer toxin is transcribed in the opposite direction than said antibody or single chain antibody gene.

In a further much preferred embodiment of the present invention said γ-toxin subunit of the *K. lactis* killer toxin lacks the signal peptide KLGT and said host

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cells are yeast cells, preferably *Saccharomyces ceri-
visiae* cells.

In still another preferred embodiment of the
present invention said target vector is introduced into
5    said host cells in linearized form. The linearization of
said target vector is preferably achieved by cutting said
first DNA sequence of said target vector at said unique
recognition site.

In a further preferred embodiment said donor
10   DNA sequence comprises a DNA sequence encoding a protein
region, preferably a CDR region of an antibody or a scFv.

The target vector and said donor sequence are
preferably introduced into said host cells by co-trans-
formation, more preferably said target vector is co-
15   transformed into said cells with a molar excess of said
donor sequence, even more preferably with at least a 25
times molar excess of said donor sequence, more prefera-
bly at least 40 times molar excess.

In yet a further preferred embodiment of the
20   present invention said yeast host cells are cultivated at
a temperature selected from the range of 24°C to 30°C,
preferably at 24°C. Said temperatures allow the selection
of host cells in which the desired replacement of the
negative selectable marker in the target vector by the
25   donor sequence has occurred.

Another aspect of the present invention re-
lates to a DNA vector which comprises the following se-
quences: a first target sequence for homologous recombi-
nation, a *TEF* promoter from Ashbya gossypii, a DNA se-
30   quence encoding at least a γ-subunit of a *K. lactis* kil-
ler toxin and a second target sequence for homologous re-
combination.

A further aspect of the present invention is
directed to a host cell, preferably a yeast cell, more
35   preferably a *S. cerevisiae* cell, comprising a DNA vector
of the present invention.

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Brief Description of the Drawings

        The invention will be better understood and
objects other than those set forth above will become ap-
5   parent when consideration is given to the following de-
tailed description thereof. Such description makes refer-
ence to the annexed drawings, wherein:
        Figure 1A shows a strategy for protein region
replacement using homologous recombination in yeast,
10      Figure 1B shows that protein region replace-
ment is highly efficient using homologous recombination,
        Figure 2A shows constructs for expression of
the γ-toxin of the *Kluyveromyces lactis* killer toxin γ-
subunit in *Saccharomyces cerevisiae*,
15      Figure 2B shows that expression of the γ-
toxin is lethal above a protein threshold and at a tem-
perature of 30°C or below,
        Figure 3 shows domain replacement strategy on
a prototype single chain using the γ-toxin as a negative
20   selection marker,
        Figure 4A shows that the use of a *PACT1::KLGT*
negative selection marker significantly reduces back-
ground growth in applications using homologous recombina-
tion, and
25      Figure 4B shows that the use of a *PTEF::KLGT*
negative selection marker significantly reduces back-
ground growth in applications using homologous recombina-
tion.


30              Modes for Carrying Out the Invention

        The method of the present invention for pro-
tein region replacement and/or randomization using ho-
mologous recombination in cells, preferably yeast cells,
35   coupled to a counterselection for non-recombining clones
offers important advantages over existing systems de-
signed for the same purpose. First, in contrast to li-

brary production using cloning or straight homologous re-
combination, the negative selection protocol keeps the
background of non-randomized clones below 0.5%, which may
be essential in certain applications. In contrast to li-
5    brary production with cloning, the library can be pro-
duced and screened in one single step in yeast. The loss
of poorly growing clones encountered during bacterial li-
brary production is therefore eliminated. In addition,
the libraries produced with homologous recombination can
10   be stored infinitely as a yeast glycerol stock, which al-
lows direct use in a next screening experiment. And, as
for libraries produced in bacteria, the libraries pro-
duced in yeast can also be used for experiments in other
organisms.

15           In the scope of the present invention it was
now found that the *Kluyveromyces lactis* killer toxin γ-
subunit (referred to as γ-toxin) can be used as a nega-
tive selection marker in a method for the generation of
randomized gene libraries. When a correct homologous re-
20   combination occurs between the target vector DNA compris-
ing the coding sequence of the γ-subunit of *K. lactis*
killer toxin and a donor sequence, the negative selection
marker is looped out thereby allowing cell survival. In
the case of vector background or non-homologous recombi-
25   nation the presence of the negative selection marker
leads to cell death

             It has been shown that the *K.* lactis killer
toxin leads to irreversible G1 arrest and loss of viabil-
ity in sensitive cells, among which are species of *Sac-*
30   *charomyces, Candida, Kluyveromyces,* and *Zygosaccharomy-*
*ces.* The α- and β-subunits of the trimeric killer toxin
are responsible for entry of the γ-subunit into sensitive
cells probably by interacting with the cell wall chitin
(Takita and Castilho-Valavicius 1993). The γ-subunit
35   alone, either when expressed extracellularly together
with the α- and β-subunits or when expressed condition-
ally intracellularly, causes the observed G1 arrest

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(Butler, White et al. 1991), (Butler, White et al. 1994)
by interfering with the function of RNA polymerase II in
a complex and still poorly understood pathway. Impor-
tantly, toxicity of the γ-subunit does not affect the
5  membrane potential, in contrast to most other killer tox-
ins that act as ionophores. Another suitable toxin for
use in the method of the present invention is the pro-
caryotic protein Kid (G. de la Cueva-Méndez et al., EMBO
J., Vol. 22, No. 2, pp.246-251, 2003).

10         The term "DNA sequence encoding a γ-subunit
of the *K. lactis* killer toxin" comprises all DNA sequence
variations which encode a functional γ-subunit i.e. a γ-
subunit which leads to loss of viability in sensitive
cells. Said term also includes functional fragments of
15  said γ-subunit.

           The term "region" as used herein encompasses
any stretch of amino acids and includes protein regions
such as e.g. protein domains, partial protein domains as
well as fragments thereof.

20         The term "antibody" as used herein includes
both intact antibody molecules and antibody fragments
(including Fab, F (ab'), Fv, and F (ab')$_2$).

           The construction of DNA vectors used in the
present invention and the generation of donor DNA se-
25  quence constructs can be done using standard molecular
biology techniques as described e.g. in Sambrook J. and
Russell D.W., Molecular Cloning: A Laboratory Manual, New
York: Cold Spring Harbor Laboratory Press, 2001).

           The length of the two target sequences flank-
30  ing the DNA sequence encoding at least the γ-subunit of
the *K. lactis* killer toxin and the homologous sequences
comprised in the donor sequence can vary. Usually, a tar-
get sequence has a length of about 35 bp to 60 bp. Said
length can depend on the specific application, specific
35  gene or regions used in a method of the present inven-
tion. The person skilled in the art is able to find the
optimal length for a specific gene or region by applying

general molecular biology methods and routine experimentation.

The method of the present invention can e.g. be used in region randomization, which is not restricted
5  to the variable regions of antibodies, but can be expanded to any protein region, in particular any small protein region. It requires that the donor DNA fragment be randomized in its central part while still preserving the sequence homologies to the target vector at its ends.
10  The randomization of the central part of the donor sequence can be done by known general molecular biology methods as e.g. described in in Sambrook J. and Russell D.W., Molecular Cloning: A Laboratory Manual, New York: Cold Spring Harbor Laboratory Press, 2001).
15      The introduction of the vector/DNA constructs described in the present invention into suitable host cells can be done by standard methods such as e.g. chemical transformation, electroporation. The host cells are cultivated under standard conditions and in standard cul-
20  ture media known to a person skilled in the art.

The invention is now further illustrated by means of examples.

**Replacement of the CDR3L of a prototype sin-**
25  **gle chain using homologous recombination in yeast**
The structure of a prototype single chain (scFv) is depicted in Figure 1A. It consists of a light VL and a heavy VH chain connected to each other with a flexible linker part. Each chain features three variable
30  regions, called CDR1, CDR2, and CDR3. Of the six CDRs, the CDR3 of the heavy chain is the major determinant of the scFv's affinity for its antigen. For generation of a high-affinity scFv, the CDR3 of VH is randomized in a library and the best binder identified in an assay. The af-
35  finity of the identified scFv is further improved by randomizing the CDR3 of VL. Randomization of the latter involves three amino acids and therefore requires a library

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complexity of 8000 independent clones. This low complex-
ity and the prospect of producing and screening the li-
brary in one step directly in yeast prompted us to ran-
domize the CDR3 of VL using homologous recombination.

5            To test the feasibility of such an idea (Fig-
ure 1A), the CDR3 of the light chain of the scFv on plas-
mid pVKS1/25 was removed using PstI and BamHI restriction
enzymes, which cut 30 and 80 bp from the CDR3 VL, respec-
tively (Figure 1A). The linearized vector was dephospho-
10    rylated using calf intestine phosphatase prior to its use
as a target sequence in the recombination experiment. The
donor DNA fragment CDR3 VL was produced by PCR using oli-
gonucleotides CDR3f_40 (Seq. Id. No. 2) and CDR3r_60
(Seq. Id. No. 3) and the scFv of pVKS1/25 as template; it
15    started 40 bp upstream of the PstI site and terminated 60
bp downstream of the BamHI site therefore providing ho-
mologies for recombination and also spanning the full
CDR3 VL sequence that had been removed in the target se-
quence. For homologous recombination, 100 ng of the line-
20    arized vector were co-transformed with a 40x molar excess
of donor DNA fragment into the yeast strain JPY9. As a
negative control, 100 ng of linearized vector without do-
nor DNA fragment were transformed. Following transforma-
tion, the cells were spread onto selective plates and in-
25    cubated at 24°C until transformants were readily visible.
The growing colonies were then counted and the number of
transformants per microgram DNA calculated (Figure 1B,
a). Co-transformation of linearized target vector and do-
nor DNA resulted in ~623'000 clones (scFv cut + CDR3L)
30    whereas transformation of linearized vector only yielded
~10'500 transformants (scFv cut) considered to be vector
background. The recombination efficiency was then calcu-
lated as the ratio of the number of transformants formed
upon co-transformation of target and donor DNA divided by
35    the number of transformants formed upon transformation of
target DNA only (Figure 1B,). It was found that the num-
ber of transformants was stimulated 59x when donor DNA

was included in the reaction. Restriction analysis of the
clones formed upon co-transformation of target and donor
DNA revealed that 37 out of 37 clones had recombined the
CDR3 VL donor DNA into the target vector, which is in
5   support of the calculated recombination efficiency. Se-
quencing confirmed that more than 95% of the analyzed
clones had performed the recombination event correctly;
the remaining 5% of the clones, even though integration
occurred at the correct site, showed one or two bp dele-
10  tions close to the recombination junctions.

        In summary, it can be concluded that integra-
tion of a donor DNA sequence into a target sequence of
choice is highly efficient. The procedure described above
can easily be adapted for randomization of a small pro-
15  tein region such as the CDR3 VL. Based on the number of
transformants formed per microgram DNA, it can be assumed
that randomization of five amino acids requiring a li-
brary with a complexity of $1.6x10^6$ clones can be achieved
using homologous recombination. In addition, the vector
20  background is in the range of 1.5% of the total clones,
which is lower than what is expected for classical li-
brary cloning.

        **Expression of the Kluyveromyces lactis killer
25  toxin γ-subunit in Saccharomyces cerevisiae**
        For some applications, it is crucial that the
vector background be as low as possible, optimally below
1%.One such example is improving the binding affinity of
a scFv for a given epitope. This is performed by random-
30  izing the CDR3 VL of a weak binder and selecting for
binders with higher affinity. Since the background clones
in such a situation are binders, even though weak ones,
they would interfere with the selection of improved bind-
ers. The inventors therefore thought of including a nega-
35  tive selection marker in the target vector DNA that would
be looped out if correct homologous recombination oc-
curred and that would result in cell death in the case of

vector background or non-homologous recombination (Figure 3). The *Kluyveromyces lactis* killer toxin γ-subunit (referred to as γ-toxin) was identified as a suitable negative selection marker. It has been shown that the *K. lac-*

5  tis killer toxin leads to irreversible G1 arrest and loss of viability in sensitive yeast cells, among which are species of *Saccharomyces*, *Candida*, *Kluyveromyces*, and *Zygosaccharomyces*. The α- and β-subunits of the trimeric killer toxin are responsible for entry of the γ-subunit

10 into sensitive cells probably by interacting with the cell wall chitin (Takita and Castilho-Valavicius 1993). The γ-subunit alone, either when expressed extracellularly together with the α- and β-subunits or when expressed conditionally intracellularly, causes the ob-

15 served G1 arrest (Butler, White et al. 1991), (Butler, White et al. 1994) by interfering with the function of RNA polymerase II in a complex and still poorly understood pathway. Importantly, toxicity of the γ-subunit does not affect the membrane potential, in contrast to

20 most other killer toxins that act as ionophores.

Prior to use of the γ-toxin as a negative selection in the recombination process, the inventors wanted to determine the sensitivity of their standard yeast strains JPY5 and JPY9 to intracellular expression

25 of the toxin. To do so, the γ-toxin lacking the signal peptide (referred to as KLGT) was cloned under control of the weak truncated constitutive *ADH1* promoter, under control of the strong constitutive *ACT1* and *TEF* promoters, and under control of the strong inducible *GAL1/10* pro-

30 moter (Figure 2A). The resulting constructs and the corresponding plasmid controls were transformed into our Wt yeast strain. Single transformants were chosen, grown in selective liquid medium, and used for serial dilution spotting assays for observing growth properties at 24°C,

35 30°C, and 37°C (Figure 2B). All plasmid controls (upper rows) grew equally well at the three temperatures tested and on either glucose or galactose (for P*GAL1/10*) selec-

tive plates. The γ-toxin expressed from PADH1tr slightly
impaired growth at 24°C without affecting growth at ei-
ther 30°C or 37°C (PADH1tr lower rows). When expressed
from the stronger PACT1, the γ-toxin completely abolished
5   growth at both 24°C and 30°C and slightly impaired growth
at 37°C (PACT1 lower rows). Using the strong PTEF from
Ashbya gossypii (Wach 1996) and the induced, strong
PGAL1/10, expression of the γ-toxin was lethal at tempera-
tures selected from the range of 24°C to 30°C and se-
10  verely reduced growth at 37°C (PTEF and PGAL1/10 lower
rows).
        It can therefore be concluded that expression
of the K. lactis γ-toxin in the yeast strains JPY5 and
JPY9 is lethal above a certain threshold protein level
15  and that the activity of the toxin is conditional allow-
ing growth at 37°C. The γ-toxin can therefore be used as
a negative marker in the recombination process.


        **Use of the killer toxin γ-subunit signifi-**
20  **cantly reduces background growth in applications using**
**homologous recombination**
        In order to make use of the γ-toxin as a
negative selection marker, the toxin under control of the
yeast ACT1 promoter was integrated into the scFv. For di-
25  rect comparison of the recombination efficiencies with
and without negative selection, the γ-toxin was inte-
grated such as to replace the CDR3 VL (Figure 3,
CDR3LΔ::PACT1::KLGT). To do so, the PstI BamHI fragment
spanning the CDR3 VL was replaced with the PACT1::KLGT
30  construct using homologous recombination in such a way
that the PstI and BamHI sites of the scFv were reconsti-
tuted. In order to avoid promoter interference with the
PACT1 driving expression of the scFv, the γ-toxin con-
struct was integrated such that it is transcribed in the
35  opposite direction of the scFv gene. Upon linearization
of the target vector within the γ-toxin sequence and upon
co-transformation of the donor DNA fragment, it is ex-

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pected that the γ-toxin is looped out in cases where proper homologous recombination occurs resulting in cell growth at 24°C. However, if either there is circular vector background or integration of the donor DNA fragment
5  does not take place, the sequence of the γ-toxin will be intact, the toxin will be expressed, and the corresponding cells will die at 24°C (Figure 3).

The effectiveness of the negative selection in, the context of homologous recombination was assayed by
10 comparing integration of the CDR3 VL donor DNA fragment into the linearized scFv target vector without negative selection to its integration into a linearized scFV target vector where the CDR3 VL had been replaced with the negative marker. The scFv including the γ-toxin negative
15 marker (scFv::PACT1::KLGT) was either linearized with the restriction enzyme PacI within the coding sequence of the toxin or with the enzyme XhoI that recognizes a site between the promoter and the toxin sequence (Figure 3). As for the scFv without negative marker, both target vectors
20 were dephosphorylated prior to use. For homologous recombination, 100 ng of each of the linearized vectors were co-transformed with a 40x molar excess of donor DNA fragment into the yeast strain JPY9. As a negative control, 100 ng of each of the linearized vectors without donor
25 DNA fragment were transformed. Following transformation, the cells were spread onto selective plates and incubated at 24°C until transformants were readily visible. The growing colonies were then counted and the number of transformants per microgram DNA calculated (Figure 4A).
30 Co-transformation of the scFv::PACT1::KLGT target linearized with PacI and the CDR3 VL donor fragment resulted in ~407'000 colonies; co-transformation of the same target vector linearized with XhoI and the CDR3 VL donor DNA yielded ~450'000 transformants. When compared to the num-
35 ber of transformants (~623'000) obtained in the absence of the toxin, a 30% reduction in the transformation efficiency was observed. Transformation of the linearized

vectors alone resulted in ~10'500, ~3'000, and ~4'500
colonies for scFv, scFv::PACT1::KLGT cut with PacI, and
for scFv::PACT1::KLGT cut with XhoI, respectively. The re-
combination efficiency increased from 59 fold without
5    negative selection to 138 or 100 fold including negative
selection depending on the site of target vector lineari-
zation. Restriction analysis of the clones produced upon
co-transformation of the target vector with negative se-
lection and the donor DNA indicated that 16 out of 16
10   clones had recombined properly. There was no difference
between the negative selection target vector linearized
at PacI or at XhoI with respect to recombination correct-
ness. In the case of target vector linearization with
PacI, restriction analysis of the vector, background
15   clones showed that closing of the vector occurred by a
recombination event at the PacI site. The result is a
four bp deletion and therefore a frameshift in the coding
sequence of the toxin. If the negative selection vector
was linearized with XhoI, it was closed by a dramatic re-
20   combination event between the actin promoter found adja-
cent to the XhoI site and the identical actin promoter
driving scFv expression. This massive recombination proc-
ess loops out the intervening γ-toxin.

        The γ-toxin has been used as a negative
25   marker for elimination of non-recombining clones in the
context of CDR3 VL replacement on a prototype single
chain. Three conclusions can be drawn from the presented
data. First, the recombination efficiency has been in-
creased by a factor 2 to 2.5 if compared to standard re-
30   combinational replacement of the same region. It was ob-
served that the recombination efficiency was higher if
the PACT1::KLGT negative marker was linearized within the
coding sequence than between the actin promoter and the
toxin sequence. This observation is explained by the fact
35   that in the latter case the actin promoter is found at
the recombinogenic ends of the linearized vector. It will
therefore recombine preferentially with the actin pro-

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moter driving expression of the scFv. This recombination
event loops out the intervening toxin allowing cell
growth without integration of the donor DNA. Such an
event is unlikely to occur upon linearization within the
5    coding sequence of the toxin because the actin promoter
driving expression of the toxin is separated from the
open, recombinogenic ends by 600 unrelated bp. However,
it was observed that the vectors linearized within the
coding sequence are closed with a minor recombination
10   event enabled by the specific sequence context around the
site of linearization. The resulting toxin lacks four bp
within the coding sequence, which renders it catalyti-
cally inactive. Cell growth at 24°C without proper inte-
gration of the donor DNA is therefore possible. Based on
15   the calculated recombination efficiencies, we can say
that the recombination event between the two identical
actin promoters takes place with a higher frequency than
the recombination event within the coding sequence of the
toxin. The second conclusion relates to the total number
20   of clones per microgram target DNA. It was seen that ~66%
of the total transformants were counted for experiments
including the negative selection if compared to region
replacements performed without negative selection. In the
latter approach, only an integration event has to take
25   place. In the former case, the integration event involves
in addition the removal of the negative selection marker,
which probably accounts for the decreased efficiency. The
third conclusion is that the fidelity of recombination is
not affected if an intervening sequence has to be looped
30   out for successful integration.

        In summary, it can be stated that using the
*K. lactis* γ-toxin as negative selection marker for coun-
teracting non-recombining clones reduces the background 2
to 2.5 fold while decreasing the total number of clones
35   produced per microgram target DNA by 30%.

Use of a heterologous promoter driving ex-
pression of the negative marker further reduces back-
ground

Even though the vector background was sig-
nificantly diminished when using a negative marker for
controlling the recombination process, the approach de-
scribed above confronted us with two problems. If the
target vector is linearized within the coding sequence of
the toxin, it can be closed without integration of the
donor DNA in a way that inactivates the toxin's catalytic
activity. This allows cell growth without occurrence of
the correct recombination event. Another problem is posed
by the finding that the actin promoters driving expres-
sion of the scFv and the toxin recombine if linearization
places the latter at the recombinogenic ends. This re-
sults in removal of the intervening toxin and as before
cell growth in the absence of a correct recombination
event.

By replacing the actin promoter of the γ-
toxin with the heterologous P*TEF* from Ashbya gossypii,
the inventors hoped to eliminate recombination between
the two promoters and thereby further decrease the back-
ground. To do so, the P*TEF::KLGT* fusion was integrated
into the prototype scFv such as to replace the CDR3 VL
(scFv::P*TEF::KLGT*) as decribed before (Figure 3). The
novel target vector was linearized as described with *Pac*I
within the coding sequence and with *Xho*I between the pro-
moter and the toxin. 100 ng of each of the linearized
vectors were co-transformed with a 40x molar excess of
donor DNA fragment into the yeast strain JPY9. As a nega-
tive control, 100 ng of each of the linearized vectors
without donor DNA fragment were transformed. Following
transformation, the cells were spread onto selective
plates and incubated at 24°C until transformants were
readily visible. The growing colonies were then counted
and the number of transformants per microgram DNA calcu-
lated (Figure 4B). Co-transformation of scFv::P*TEF::KLGT*

linearized with *PacI* and the donor CDR3 VL DNA yielded ~470'000 clones; co-transformation of the donor DNA and the same vector linearized with *XhoI* produced ~360'000 transformants. The control transformation using the line-
5   arized scFv and the donor DNA resulted in ~623'000 colo-nies. The recombination efficiency was 115 when using the target vector linearized within the coding sequence of the toxin and 302 for use of the target vector linearized between the heterologous promoter and the toxin. When
10  compared to the recombination efficiency obtained with the target vector without the γ-toxin, a two- up to al-most 6 fold increase was observed. Restriction analysis of the clones produced in the co-transformation experi-ments showed that 22 out of 22 clones had looped out the
15  toxin and integrated the CDR3 VL donor DNA at the correct site. Fidelity of the recombination event was not af-fected regardless of whether the negative selection tar-get vector was linearized with *PacI* or with *XhoI*. Analy-sis of the background clones produced upon transformation
20  of scFv:: P*TEF*::*KLGT* linearized with *PacI* indicated clos-ing of the vector by a minor non-homologous recombination event involving the *Pac* I sequence as seen for scFv:: P*ACT1*::*KLGT* under the same conditions. If the negative se-lection vector was linearized with *XhoI*, it was closed by
25  a dramatic recombination event between the *TEF* promoter found adjacent to the *XhoI* site and a related sequence in some other part of the vector. As a result, the γ-toxin is removed without integration of the donor DNA.

Region replacement was performed using ho-
30  mologous recombination. The *Kluyveromyces lactis* killer toxin γ-subunit was introduced at the site to be replaced as a negative selection marker. In an attempt to further minimize growth of background clones, the heterologous promoter P*TEF* was used for driving expression of the γ-
35  toxin instead of an endogenous *S. cerevisiae* promoter. The target vector was then linearized either within the coding sequence of the toxin or between the promoter and

the toxin for co-transformation with the CDR3 VL donor
DNA. It was found that linearization within the coding
sequence resulted in a similar recombination efficiency
for the actin and the *TEF* promoter driving expression of
5    the toxin. Closing of the vector without integration of
the donor DNA and therefore appearance of the background
clones was achieved in both cases with the identical re-
combination event at the *PacI* site, which results in a
frameshift within the coding sequence of the toxin. This
10   supports the notion that the recombinogenic ends of the
linear fragment determine mainly the sites of recombina-
tion and not the promoters located 600 bp downstream. If
the target vector was linearized between the promoter and
the toxin, a recombination efficiency of ~300 was ob-
15   served for use of the heterologous promoter compared to
one of ~100 with the *S. cerevisiae* endogenous actin pro-
moter. Restriction analysis of the rare background clones
produced upon transformation of the target vector line-
arized between the *TEF* promoter and the toxin indicated
20   that growth was enabled because of a dramatic recombina-
tion event between one of the linear ends and a related
sequence in the vector. Based on the recombination effi-
ciencies, it can be concluded that the illegitimate re-
combination event for closing of the vector bearing the
25   *TEF* promoter is significantly less frequent than intra-
plasmid recombination between the two actin promoters.
Together with the fact that fidelity of recombination is
not affected by use of the *TEF* promoter, it should be em-
phasized that background in region replacements using ho-
30   mologous recombination is minimal (less than 0.5%) if the
γ-toxin negative selection marker is under control of the
heterologous *TEF* promoter and if the target vector is
linearized between the *TEF* promoter and the γ-toxin.
        In the scope of the present invention, ho-
35   mologous recombination has been used for replacement of
the CDR3 VL of a prototype single chain. In contrast to
other studies (Hua, Luo et al. 1998), (Zhu and Hua 2002),

the *Kluyveromyces lactis* killer toxin γ-subunit has been included in the target vector to counterselect clones that do not integrate the donor DNA correctly. The negative marker was inserted into the target vector instead

5  of the sequence to be replaced. The linearized target vector and the accordingly designed donor DNA were co-transformed into a Wt yeast strain, the total number of transformants per microgram vector DNA and the recombination efficiency calculated. The numbers were determined

10  for target vectors with the γ-toxin under control of either an endogenous *S cerevisiae* or a heterologous promoter and compared to homologous recombination without the negative selection. It was found that the maximal number of transformants was decreased from 620'000 to

15  ~400'000 when the negative marker was included. On the other hand, the recombination efficiency was increased from 59 to 138 for use of the endogenous yeast promoter and to 302 in combination of the γ-toxin with the heterologous *TEF* promoter from *Ashbya gossypii*. On one hand,

20  these data show that homologous recombination is highly efficient and can be used for production of libraries with a complexity of few millions of independent clones. On the other hand, the results clearly demonstrate the usefulness of the γ-toxin as a negative selection marker

25  for minimizing the number of background clones. They also emphasize the importance of using a heterologous promoter for expression of the γ-toxin. It should be noted that not only the *TEF* promoter is suitable for this application, but that any other strong heterologous promoter

30  functional in *S. cerevisiae* will serve the purpose. With use of a heterologous promoter, background can be kept as low as 0.3%, which is significantly below what is expected for either traditional library production with cloning (5%) or library production using straight homolo-

35  gous recombination (1.7% in our hands). In order to facilitate experimental handling, use of the uncut, circular vector as target sequence can be envisioned.

21

## Materials and Methods

### Yeast media, strains, and genetic techniques

5      Strains without plasmids were grown in complete medium YPAD (2% (w/v) glucose, 2% (w/v) peptone, 1% (w/v) yeast extract, 40 µg/ml adenine sulfate, 2% (w/v) agar for solid medium). Strains bearing plasmids were selected on yeast nitrogen base (YNB) minimal medium containing the required nutritional supplements (Guthrie and Fink 2002). For galactose induction experiments, selective plates containing 2% (w/v) galactose, 2% (w/v) raffinose, 1% (w/v) glycerol, and the required nutritional supplements were used (Guthrie and Fink 2002). Standard transformation of yeast cells was accomplished using the high efficiency lithium acetate method (Woods and Gietz 2001).

      The yeast strains used were JPY5 (*Matα leu2 ura3 trp1 his3 lys2*) (Barberis, Pearlberg et al. 1995) for galactose induction experiments and JPY9 (*Matα leu2 ura3 trp1 his3 lys2 gal4Δ*) (Barberis, Pearlberg et al. 1995) for all other applications.

### DNA techniques and plasmid constructions

25     All DNA manipulations were performed according to standard techniques (Sambrook and Russell 2001). Restriction enzymes, calf intestine phosphatase (CIP), T4 DNA polymerase, and T4 DNA ligase were purchased from New England Biolabs or Invitrogen. All PCRs for cloning purposes were performed with a DNA polymerase with proofreading activity (Vent, New England Biolabs). Purification of DNA fragments was performed with the QIAquick Gel Extraction kit from Qiagen; plasmids were purified using the Wizard Plus Miniprep kit from Promega. Oligonucleotides were synthesized by Microsynth GmbH and by Invitrogen.

        The γ-subunit of the *Kluyveromyces lactis*
killer toxin (γ-toxin) was cloned as four separate frag-
ments based on the presence of suitable restriction
sites. Three of the four fragments were either produced
5  by annealing of complementary oligonucleotides followed
by cloning into pBS or by annealing of partially overlap-
ping oligonucleotides followed by a fill-in reaction with
T4 DNA polymerase and subsequent cloning into pBS. For
production of the fourth fragment, three independent,
10 partially overlapping smaller fragments were produced by
annealing of oligonucleotides followed by a fill-in reac-
tion with T4 DNA polymerase. The resulting three frag-
ments were then co-transformed into yeast together with a
target vector of choice resulting in generation of the
15 larger fragment by homologous recombination. For produc-
tion of pCS116, the four fragments were removed from
their original cloning vectors with restriction enzymes
present within the coding sequence of the γ-toxin and
cloned sequentially into pBS such as to preserve the cod-
20 ing sequence. The γ-toxin lacking the signal peptide was
amplified from pCS116 with a 5' oligonucleotide bearing
an *Xho*I site and a 3' oligonucleotide bearing a *Not*I site
for cloning into pMH5 resulting in pCS118. For construc-
tion of pCS131, pCS133, and pCS135, the γ-toxin together
25 with T*GAL11* was released from pCS118 with *Xho*I and *Hind*III
and cloned into pMH3, pMH4, or pDU10, respectively. P*TEF*
was amplified from pFA6a-*kanMX4* (Wach *et al.*) with a 5'
oligonucleotide bearing an *Eco*RI site and a 3' oligonu-
cleotide bearing an *Xba*I site and cloned into pMH5 such
30 as to replace P*ADHfl* resulting in plasmid pCS137. For con-
struction of pCS140, the γ-toxin together with T*GAL11* was
released from pCS118 with *Xho*I and *Hind*III and cloned
into pCS137. For generation of pCS136 and pCS143, the γ-
toxin together with the corresponding promoter was ampli-
35 fied with a 5' oligonucleotide bearing homologies to the
region upstream of the *Pst*I site found immediately 5' of
the CDR3L and to the C-terminus of the γ-toxin and a 3'

oligonucleotide bearing homologies to the corresponding
promoter and to the region downstream of the *Bam*HI site
found immediately 3' of the CDR3L. The such generated PCR
fragment was co-transformed with pVKS1/25 linearized with
5   *Pst*I and *Bam*HI into yeast, and clones growing at 37°C,
but not at 24°C, were selected. Correct integration of
the promoter/γ-toxin fusion was confirmed by restriction
analysis and sequencing.

10          **Homologous recombination and analysis of re-
sulting clones**
            For the purpose of homologous recombination,
the target vector of choice was linearized with the indi-
cated restriction enzyme(s), treated with CIP; and puri-
15  fied over a Qiagen DNA column. The donor fragment used
for homologous recombination was designed on one hand
such as to include the *Pst*I – *Bam*HI sequence of the sin-
gle chain (scFv) light chain, which .spans the CDR3. On
the other hand, 40 basepairs (bp) or 60 bp homologous to
20  the scFv sequence immediately 5' of the *Pst*I site or im-
mediately 3' of the *Bam*HI site, respectively, were added
to the 5' and to the 3' end of the donor fragment for ef-
ficient homologous recombination. The donor fragment was
generated by PCR using oligonucleotides CDR3f_40 (5' CAG
25  TGG ATC TGG GAC AGA AT) (Seq. Id. No. 1) and CDR3r_60 (5'
GAG TCT CAG GGA CCC CCC AG) (Seq. Id. No. 2) and pVKS1/25
as template. The resulting product was digested with *Dpn*I
to remove parental DNA and purified over a Qiagen DNA
column. The concentration of both target vector(s) and
30  donor PCR fragment was determined on an agarose gel using
a marker reference (2-log DNA ladder, New England Bio-
labs). For co-transformation of target and donor DNA into
yeast, 100 µg of target vector and a 40x molar excess of
PCR donor fragment were combined. When used for homolo-
35  gous recombination, an optimized high efficiency lithium
acetate transformation protocol was used. The strain JPY9
was harvested at a cell density of $4\text{x}10^7$. Resuspension

volumes were adjusted accordingly, and 50 µl of the cells competent for transformation were used for each single transformation. Heat shock time was increased to 40 minutes. All other parameters were according to (Woods and

5  Gietz 2001).

For analysis of the growing clones, a PCR with oligonucleotides CDR3f_40 (Seq. Id. No. 1) and CDR3r_60 (Seq. Id. No. 2) was performed directly on the yeast clones. The resulting PCR product was on one hand

10  analyzed on an agarose gel and on the other hand subjected to restriction analysis with PstI and BamHI. This procedure allowed a direct assessment of the recombination event and the sequence at the recombination junction. In addition, plasmids were rescued from individual

15  transformants and the region modified during homologous recombination was sequenced.


**Spotting assay**

Strains were grown to saturation in selective

20  medium at 37°C and cell density was determined. The cultures were then diluted to $5\times10^6$ cells/ml, $1.5\times10^6$ cells/ml, $5\times10^5$ cells/ml, $1.5\times10^5$ cells/ml, and $5\times10^4$ cells/ml in sterile water. 5 µl of each serial dilution corresponding to 25'000, 7575, 2500, 757, or 250 cells,

25  respectively, were spotted onto selective glucose and/or galactose plates. Plates were then incubated at 24°C for two days, at 30°C for 1.5 days, and at 37°C for 1 day.

While there are shown and described presently preferred embodiments of the invention, it is to be dis-

30  tinctly understood that the invention is not limited thereto but may be otherwise variously embodied and practiced within the scope of the following claims.

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## References

Barberis, A., J. Pearlberg, et al. (1995). "Contact with a component of the polymerase II holoenzyme suffices for gene activation." Cell 81(3): 359-68.

Butler, A. R., J. H. White, et al. (1994). "Two Saccharomyces cerevisiae genes which control sensitivity to G1 arrest induced by Kluyveromyces lactis toxin." Mol Cell Biol 14(9): 6306-16.

Butler, A. R., J. H. White, et al. (1991). "Analysis of the response of Saccharomyces cerevisiae cells to Kluyveromyces lactis toxin." J Gen Microbiol 137(Pt 7): 1749-57.

Guthrie, C. and G. R. Fink (2002). Guide to yeast genetics and molecular and cell biology. San Diego, Academic Press.

Hua, S. B., Y. Luo, et al. (1998). "Construction of a modular yeast two-hybrid cDNA library from human EST clones for the human genome protein linkage map." Gene 215(1): 143-52.

Sambrook, J. and D. W. Russell (2001). Molecular Cloning: a laboratory manual. New York, Cold Spring Harbor Laboratory Press.

Takita, M. A. and B. Castilho-Valavicius (1993). "Absence of cell wall chitin in Saccharomyces cerevisiae leads to resistance to Kluyveromyces lactis killer toxin." Yeast 9(6): 589-98.

Wach, A. (1996). "PCR-synthesis of marker cassettes with long flanking homology regions for gene disruptions in S. cerevisiae." Yeast 12(3): 259-65.

Woods, R. A. and R. D. Gietz (2001). "High-efficiency transformation of plasmid DNA into yeast." Methods Mol Biol 177: 85-97.

Zhu, L. and S. B. Hua (2002). Generation of highly diverse library of expression vectors via homologous recombination in yeast. United States Patent No. 6,410,271.